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Evaluation of Cytotoxic, Membrane Damaging and Apoptotic Effects of *Origanum majorana* Essential Oil on Lung Cancer and Epidermoid Carcinoma Cells

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ABSTRACT

BACKGROUND/AIMS: In this study, the potential of an essential oil to cause membrane damaging and induce apoptosis was investigated in order to determine the cytotoxic effect of the essential oil obtained from *Origanum majorana* (*O. majorana*), popularly known as "sweet marjoram", on lung cancer (A-549) and epidermoid carcinoma (A-431) cells.

MATERIALS AND METHODS: In order to determine the cytotoxic dose and IC_{50} value of an essential oil, the essential oil was applied to cells in the concentration range of 5-500 µg/mL for 24, 48 and 72 hours. After those applications, CellTiter-Blue® Cell Viability Assay and 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT) assay were performed on the cell lines. The lactate dehydrogenase (LDH) Activity test was used to determine the membrane damaging effect of the essential oil on A-549 and A-431 cells. Cytotoxicity experiments were followed up by caspase-3/7 activity assays to get a mechanistic insight into the associated molecular pathways.

RESULTS: It was observed that treatments of essential oil from *O. majorana* on A-549 and A-431 cells inhibited cell proliferation, that is, it had a cytotoxic effect and caused an increase in LDH activity, which is one of the membranes damaging markers, and caspase-3/7 activity, which is one of the enzymes involved in the apoptotic pathway, in both cells in comparison to controls at the end of the 24-hour incubation.

CONCLUSION: The results of this study indicate that the increase in LDH activity and caspase-3/7 activity caused by *O. majorana* essential oil treatment reveal the membrane damaging and apoptotic effects, respectively. *O. majorana* essential oil can be evaluated as a potential therapeutic agent for lung cancer and epidermoid carcinoma due to these effects. However, more research is needed to confirm this.

Keywords: *Origanum majorana*, essential oil, apoptotic effect, membrane damage

INTRODUCTION

Currently, chemotherapy treatment with existing drugs is insufficient and treatment cannot be provided in many types of cancer. Therefore, studies to develop new anti-cancer drugs appear as a promising field. Cancer researchers, who aim to enable treatment in cancer, focus on developing new agents/drugs with strong anti-cancer effects. In recent years, the use of plants as drugs in cancer treatment has become widespread due to their promising effects against a wide variety of tumor cell lines.

As a result of the great variety in Turkey's ecology, it has many medicinal and aromatic plants and, in parallel, approximately 30% of the species in its flora are aromatic plants. Aromatic plants are the main source of essential oils.[1] Approximately three thousand essential oils are known and three hundred of them are commercially important today.

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These are also widely used in the fields of food, cosmetics, health and perfumery.[2]

Many drugs with known antineoplastic properties are originated from plants. For example, many anticancer drugs used today such as vinblastine, irinotecan, topotecan, and vincristine are obtained from plants. In this context, it is important both scientifically and economically to obtain and evaluate medicinal plants and their essential oils in their pure form.

Researching new treatment strategies that use natural components obtained from plants against cancer, which is one of the most important diseases of our age, has become one of the most important issues today. Due to their structure, essential oils can easily interact with the cell walls and membranes and thus they damage the cell membrane. Additionally, essential oils can cause ionic mitochondrial membrane depolarization in eukaryotic cells. They decrease the membrane potential by affecting the Ca^{2+} cycle and other ionic channels.[2,3]

The most scientifically researched aromatic plant is thyme. The genera, including the thyme species that are traded and widely used in Turkey, are *Origanum*, *Thymbra*, *Coridothymus*, *Satureja* and *Thymus*.

Studies have shown that the *Origanum* species have antibacterial, antifungal, antiviral, antioxidant, anti-inflammatory, antiplatelet, anticarcinogenic, cytotoxic, antidiabetic, acetylcholinesterase and butyrylcholinesterase inhibitor, and insecticidal effects. In addition, it has traditionally been used for gastrointestinal disorders. This genus is also known to be rich in essential oils.[4]

Origanum majorana L. (Lamiaceae) (*O. majorana*), also known as "sweet marjoram", has been used effectively as a spice since ancient times.[5] It is in the "generally recognized as safe (GRAS)" category due to its use in the treatment of different diseases.[6] In another study, the instrumental analysis of *O. majorana* L. essential oil was performed using the GC-MS method, and thus the components and ratios of the essential oil were determined. Carvacrol (52.50%) and linalool (45.4%) were determined to be the two compounds with the highest ratio.[7]

Apoptosis is one of the most frequently used mechanisms in the development of anticancer drugs. The mechanisms regulating apoptosis in the cell are highly complex.[8] One of the most important events linked to the apoptosis mechanism is caspase activation.[9]

The cytotoxic and apoptotic effects of *O. majorana* essential oil, which we think may be a new agent candidate that can be used in cancer treatment, in A-549 (wild-type p53) and A-431 (mutant p53) cells were investigated. Thus, we aimed to investigate whether or not this essential oil has any effect on p53 status according to the response of the cells.

MATERIALS AND METHODS

Plant Material Used

The essential oil to be used in the studies was obtained from *O. majorana* collected from Turkey (Alanya-730 m). The collected plant samples were dried under suitable conditions. The classification of the collected plant samples was made by experts working in this field.

Obtaining the Essential Oil

The essential oil was obtained after the dried *O. majorana* to be used in the study was kept under suitable conditions. The root parts of the plant were separated and the essential oil was obtained from the remaining stem parts (aerial parts) (100 g). 100 g of the plant was weighed and placed in flask for 3 hours and pure water was added to the plant. The essential oil was obtained by a steam distillation method in Clevenger apparatus. The essential oils obtained were kept at +4 °C until they were to be used in the study.

Cell Culture

The cells used in this study (A-549-wild-type p53, A-431-mutant p53) were obtained from the American Type Culture Collection (ATCC). The content of the cell culture medium we used in our experiments was 10% fetal bovine serum constituted RPMI supplemented with 1% penicillin-streptomycin.

Cell Viability Assays

After the essential oil was dissolved with dimethylsulfoxide (DMSO), it was diluted with the medium and added to the wells containing the cells in the concentration range of 5–500 µg/mL and incubated for 24, 48 and 72 hours. After incubation, the viability of the cells was measured with Cell Titer-Blue® cell viability assay and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Resazurin, the active ingredient of Cell Titer-Blue® cell viability assay, is a non-toxic, blue-colored, non-fluorescent compound that can pass through the cell membrane. Once inside the cell, it is reduced to resofurin, which fluoresces strongly red. It has an important sensitivity due to its fluorescent feature.[10]

MTT assay is a method in which the amount of cell proliferation is determined based on the colorimetric measurement of formazan dyes or enzymatic activity due to MTT reduction. The method is based on the principle of colorimetric determination of color change in cells incubated with the MTT agent. The MTT method was applied after the cells were treated with the essential oil for 24, 48 and 72 hours. After incubation, absorbance values were measured at 490 nm.[11] The 50% inhibitory concentration (IC_{50}) value was calculated by determining the % cell viability curve with the help of the Microsoft Excel program. The tests were repeated three times.

Measurement of Lactate Dehydrogenase (LDH) Release

With the LDH activity assay, one of the methods used in the evaluation of cell death, the activity of the cytoplasmic enzyme released from dead or damaged cells is measured when the integrity of the cell membrane is disrupted. LDH, a stable cytoplasmic enzyme found in all cells, can easily be released into the cell culture supernatant when the cell plasma membrane is damaged.

Changes in LDH enzyme activity were determined by applying the essential oil (IC_{50}) to the cells for 24 hours. LDH enzyme activity was determined by the method specified in the "Lactate Dehydrogenase Activity Assay Kit" (MAK066) produced by Sigma-Aldrich. Measurements were made in triplicate.

Caspase-3/7 Activity

One of the caspases that plays a key role in apoptotic signaling is caspase 3/7. Caspase 3/7 is the main caspase that gains activity in the early stages of apoptotic cell death and causes death to occur. Caspase 3/7 enzyme activity was determined by the method specified in the "ApoTox-Glo™ Triplex Assay" kit produced by Promega after the

treatment of *O. majorana* essential oil (IC_{50}) for 24 hours. Cell lysis occurs with the addition of the reagent, which is one of the kit components, to the medium. Cleavage of the substrate by caspase is followed by the generation of a "sparkling" luminescence signal produced by luciferase. The luminescence that occurs is proportional to the amount of caspase activity present. The experiments were performed in triplicate.

Statistical Analysis

The values of the control and test samples were given with mean and standard error (SE). Statistical analysis of the obtained data was made with Minitab 18 program (State College, PE, USA) (DC Montgomery, 2001). Analysis of variance (ANOVA) was used for comparison between the means of three or more independent (unrelated) groups according to some variable. Tukey's multiple comparison test was applied to indicate the significance level of the differences. Statistical differences were considered significant at $p<0.05$.[12]

RESULTS

Determination of the Cytotoxic Effect of *O. Majorana* Essential Oil on A-549 and A-431 Cells

The cytotoxic effects of essential oils obtained from *O. majorana* on the A-549 and A-431 cell lines were measured using the MTT and Cell Titer-Blue® cell viability assays. After the cells were incubated with the essential oil, the IC_{50} (the concentration that kills 50% of the cells) and IC_{70} (the concentration that kills 70% of the cells) values of the essential oil were calculated for each cell (Table 1). According to the cytotoxicity results we obtained, we determined that the Cell Titer-Blue® cell viability assay was more sensitive than the MTT assay. For this reason, we made evaluations according to the concentrations we calculated using the data obtained from the CellTiter-Blue cell viability assay in the other parameters we studied. According to both assays, it was observed that the cytotoxic effect increased in parallel with increasing concentrations and incubation times in both A-431 cells and A549 cells (Figures 1 and 2). According to the MTT assay, after 24, 48 and 72 hours essential oil incubations, IC_{50} values were calculated to be 266, 222 and 182 µg/mL in A-549 cells, and 218, 187 and 140 µg/mL in A-431 cells, respectively (Table 1). In addition, according to the CellTiter-Blue® Cell Viability assay, after 24, 48 and 72 hours of essential oil incubation, the IC_{50} values of cells were calculated to be 243, 203 and 167 µg/mL in A-549 cells and 182, 145 and 111 µg/mL in A-431 cells, respectively (Table 1). These results showed that the incubation time affected the essential oil's cytotoxic effects on both A-549 and A-431 cells. The essential oil was found to be more effective on A-431 cells (mutant p53) than A-549 cells (wild-type p53). It was found that DMSO (0.5%, v/v), which we used to dissolve the essential oil, did not affect the cell viability in either A-431 or A-549 cells when treated for the same time periods.

Lactate Dehydrogenase (LDH) Activity Measurement

Lactate dehydrogenase (LDH) is the enzyme that converts lactic acid to pyruvic acid in the presence of NAD^+. Lactic acid occurs with the disruption of cell integrity. That is, with the death of cultured cells, the integrity of the cell membrane is disrupted and the contents of the cytoplasm come out. Since the lactate dehydrogenase enzyme is usually found in every cell, it helps in determining membrane damage depending on the enzyme activity.[13] To evaluate the effect of *O. majorana* essential oil on the cell membrane, LDH activity, which is also a marker of early apoptosis and released from cells into the medium, was measured.[14]

After the application of the essential oil at IC_{50} concentration to A-549 and A-431 cells for 24 hours, LDH activities in the cells were determined

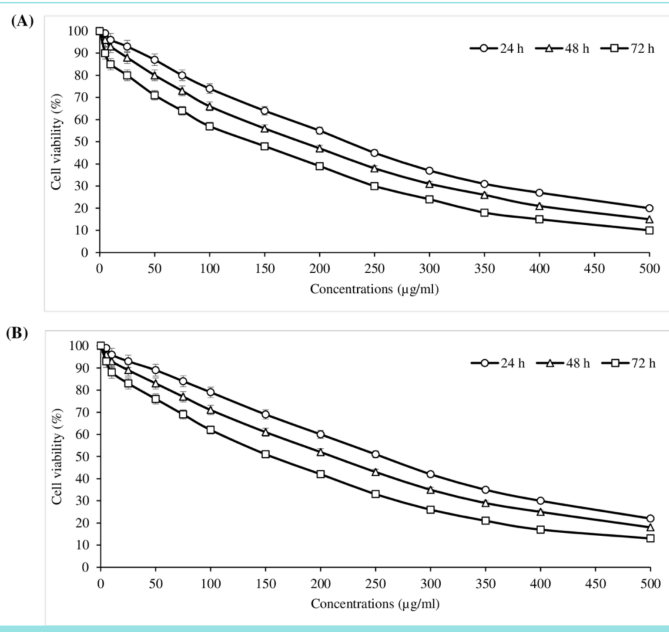

Figure 1. The cytotoxic effects of *O. majorana* essential oil on A-549 cells after 24, 48 and 72 hours measured by **(A)** The CellTiter-Blue-Cell Viability Assay; **(B)** MTT Assay. Results are presented as viability ratio compared with the control group (treated with only the medium-untreated cells). Values are expressed as the mean of three separate experiments ± SE. Error bars represent standard error of the mean from three replications.

SE: standard error

Table 1. Summary of the cytotoxic effects of *O. majorana* Essential oils on A-549 and A-431 cells

Cells treatments	Es. oil (µg/mL) (CellTiter.) X ± SE	Es. oil (µg/mL) (MTT) X ± SE
A-549, 24 h, IC_{50}	243±2.99$^{\prime\prime\prime\prime}$	266±2.32$^{\text{ñ}}$
A-549, 24 h, IC_{70}	348±3.66*	377±2.13$^{\varepsilon}$
A-549, 48 h, IC_{50}	203±4.00$^{\text{£}}$	222±3.45$^{\text{£}}$
A-549, 48 h, IC_{70}	298±3.71$^{\#}$	322±2.88‡
A-549, 72 h, IC_{50}	167±2.21§	182±2.34$^{\text{€}}$
A-549, 72 h, IC_{70}	259±2.34$^{\text{ñ}}$	274±2.66$^{\text{ñ}}$
A-431, 24 h, IC_{50}	182±3.03$^{\text{€}}$	218±2.78$^{\text{£}}$
A-431, 24 h, IC_{70}	300±2.88$^{\#}$	317±2.31‡
A-431, 48 h, IC_{50}	145±2.66$^{\$}$	187±3.03$^{\text{€}}$
A-431, 48 h, IC_{70}	253±3.72$^{\text{ñ}}$	283±2.99$^{\#}$
A-431, 72 h, IC_{50}	111±2.41*	140±2.88$^{\$}$
A-431, 72 h, IC_{70}	212±3.05$^{\text{£}}$	223±2.76$^{\text{£}}$

Values followed by different asterisks within a column are significantly different ($p\leq0.05$).
SE: standard error, Es. oil: essential oil, CellTiter: CellTiter-Blue cell viability assay. X is an average of five repetitions.

(Figure 3). It was observed that LDH enzyme activity increased 4.8 times in A-549 cells and 5.4 times in A-431 cells to which IC_{50} essential oil concentrations were applied, compared to the control group cells (only treated in the medium).

LDH activity in A-431 cells increased to a greater extent in comparison to LDH activity in A-549 cells, and LHD activity in both A-431 and A-549 cells was higher compared to the control group. LDH enzyme activity in A-431 cells was statistically different when compared to the LDH enzyme activity in A-549 cells ($p<0.05$).

Measuring the Effects of Essential Oil on Caspase-3/7 Enzyme Activity in A-549 and A-431 Cells

The apoptosis inducing potential of the essential oil was observed after 24 hours of *O. majorana* essential oil treatment on A-549 cells and A-431 cells. Caspase-3/7 activity in A-431 cells increased 2-fold at the end of 24 hours incubation, while caspase-3/7 activity in A-549 cells increased 4-fold (Figure 4). It was observed that the apoptotic effect of the essential oil, in terms of increasing the caspase-3/7 activity, in A-549 cells was two times higher than the apoptotic effect of the essential oil in A-431 cells. After the essential oil treatment, the highest increase in caspase-3/7 activity was observed in A-549 cells compared to the control.

The LDH and caspase 3/7 activity observed after the essential oil treatments was statistically different from the control groups (treated with only the medium-untreated cells) in all cell lines ($p<0.05$). The LDH and caspase 3/7 activity of essential oil treatments in A-549 was statistically different from the LDH and caspase 3/7 activity of essential oil treatments in A-431 ($p<0.05$).

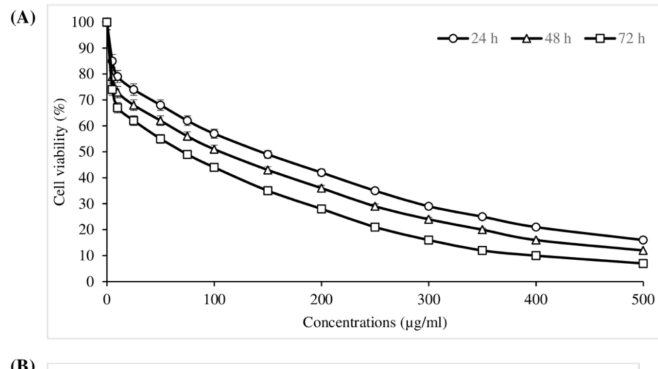

Figure 2. The cytotoxic effects of *O. majorana* essential oil on A-431 cells after 24, 48 and 72 hours measured by **(A)** The CellTiter-Blue-Cell Viability Assay; **(B)** MTT Assay. Results are presented as viability ratio compared with the control group (treated with only the medium-untreated cells). Values are expressed as the mean of three separate experiments ± SE. Error bars represent standard error of the mean from three replications.

SE: standard error

DISCUSSION

Lung and epidermoid cancers are among the most common types of cancer seen all over the world. Chemotherapy is an important strategy used in the treatment of lung and epidermoid cancers. However, the

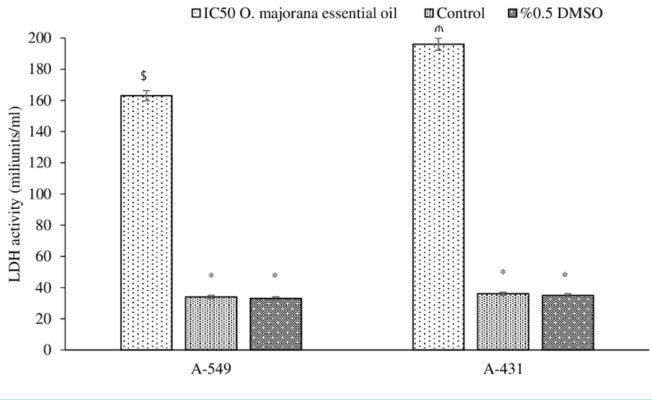

Figure 3. Lactate dehydrogenase activities in A-549 and A-431 cells after being treated with *O. majorana* essential oil. Values are expressed as the mean of three separate experiments ± SE. Error bars represent standard error of the mean from three replications, and bars with the same asterisks indicate no significant difference (ANOVA with Tukey's test, $p\leq0.05$). Different asterisks represent significant differences among treatments (ANOVA, $p\leq0.05$) in A-549 and A-431 cells. One unit of LDH activity is defined as the amount of enzyme that catalyzes the conversion of lactate into pyruvate to generate 1.0 µmole of NADH per minute at 37 °C.

SE: standard error, LDH: lactate dehydrogenase

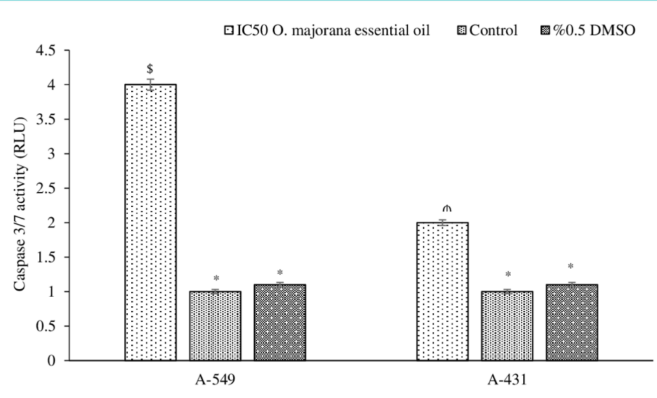

Figure 4. Effect of *O. majorana* essential oil on caspase-3/7 activity in A-549 and A-431 cells. Values are expressed as the mean of three separate experiments ± SE. Error bars represent standard error of the mean from three replications, and bars with the same asterisks indicate no significant difference (ANOVA with Tukey's test, $p\leq0.05$). Different asterisks represent significant differences among treatments (ANOVA, $p\leq0.05$) in A-549 and A-431 cells.

SE: standard error, RLU: relative light unit

desired success has not been obtained from chemotherapy to date. Therefore, it is desired today to use therapeutically effective compounds that can be obtained from natural sources with little or no adverse effects on healthy cells in the treatment of cancer. In this respect, it is very important to evaluate the therapeutic effects of natural resources in cancer research.

O. majorana, which belongs to the Lamiacea family, has attracted the attention of many scientists due to its high essential oil potential. *O. majorana* is one of the most important aromatic plants that contain major antioxidants such as flavonoids and triterpenoids.[15] Phenolic acids and flavonoids have been reported to play a role in the prevention of human pathologies.[16] The Food and Drug Administration regard *O. majorana* to be generally safe.

Dose and time dependent inhibition by *O. majorana* essential oil and linalool were observed with IC_{50} values of 100, 80 and 63 µg/mL for essential oil and 81.5, 72.7 and 64.7 µg/mL at 24, 48 and 72 hours, respectively on Hep G2 cells as assessed by CellTiterBlue® Cell Viability Assay.[7] In that study, the results showed that incubation time affected the cytotoxic effects of the essential oil and linalool on Hep G2 cells. In another study, *O. majorana* essential oil and linalool were found cytotoxic in terms of concentration and time-dependence in both parental and epirubicin-resistant (drug resistant) H1299 cells. The cytotoxic effects of *O. majorana* essential oil for 24, 48 and 72 hours were found to be higher than linalool in both parental and drug-resistant cells. Also, it was observed that *O. majorana* essential oil and linalool were less cytotoxic on drug resistant cells than parental cells. In other studies, eugenol, eucalyptol, terpinen-4-ol camphor, carvacrol and thymol, which are found as components in many essential oils, showed more cytotoxicity in parental H1299 cells than epirubicin-HCl resistant H1299 cells.[17,18] The essential oils from wild and cultivated forms of *Salvia pisidica* showed cytotoxicity on H1299 cells.[19]

LDH, one of the important enzymes of the glycolytic pathway, is found in the cytoplasm of all cells. LDH is rapidly released into the cell culture medium when the plasma membrane is damaged. An increase in the number of dead or plasma membrane-damaged cells results in increased LDH activity in the culture supernatant.[13] In our study, we tried to reveal the membrane damaging effects of *O. majorana* essential oil by determining the changes in LDH activity after treatment of IC_{50} *O. majorana* essential oil on cells for 24 hours. It was found that the LDH enzyme activity increased with respect to control cells in cells subjected to *O. majorana* essential oil treatment. Our LDH activity result supports cell viability results. In our previous study, *O. majorana* essential oil and its oxygenated monoterpene component linalool resulted in increased malondialdehyde levels on both parental and drug resistant H1299 cells.[20] Also, eugenol, eucalyptol, terpinen-4-ol, camphor, carvacrol and thymol, which are found as components in many essential oils, showed membrane damaging effects in parental and epirubicin-HCl resistant H1299 cells.[17,18] The essential oil of *Origanum onites* (Lamiaceae) and its two phenolic components, thymol and carvacrol increased MDA levels with respect to controls on Hep G2 cells.[21] Some plant essential oils showed membrane damaging effects in a dose dependent manner on cancer cells.[22]

In our study, caspase-3/7 activity, one of the apoptotic death markers, was measured in A-431 cells and A-549 cells after essential oil treatment. Our results suggest that *O. majorana* essential oil induced apoptosis in all cells. It has been reported that a water extract of *O. acutidens* caused cytotoxic and apoptotic effects in breast cancer cells such as MCF-7, MDA-MB-468 and MDA-MB-231. This apoptotic effect was demonstrated by showing that caspase-7 protein expression and the number of TUNEL-positive cells increased at the end of the application.[23] In our study, it was demonstrated that *O. majorana* essential oil caused higher caspase-3 activity in both cells compared to the control cells, and it was revealed that the essential oil induced intense apoptotic effect in the cells. As a result, *O. majorana* essential oil treatment induced apoptosis in both cells.

When the cytotoxic effects of *O. majorana* essential oil in A-549 and A-431 cells were compared in our study, it was observed that this essential oil was more effective in A-431 cells. The reason why the essential oil has different cytotoxic effects on these cells may be due to the different properties of the cells. A-549 has functional p53 and EGFR copy number of 3.4, and A-431 cell line has mutant p53 and expresses EGFR in high amounts. The results obtained from our LDH activity experiment support our cytotoxicity results. In addition, it has been demonstrated that this essential oil exerts its cytotoxic effect by stimulating the apoptotic mechanisms in both cells. The new experiences/gains we have obtained as a result of our study may lead to the evaluation of plant-derived compounds in epidermoid and lung cancer studies.

Limitations of the Study

There are some limitations to our study. Firstly, despite essential oils very important natural bioactive properties, they have certain disadvantages such as hydrophobic behavior in aqueous environments, losses due to their volatile properties, and rapid consumption of their bioactive properties due to uncontrolled activity. In addition, undesirable changes may occur in their chemical compositions over time, due to the fact that they are quickly affected by factors, depending on their storage conditions, such as oxygen, humidity, temperature, and light. For all these reasons, we should have used different methods to ensure the stability of essential oils and to protect and/or increase their bioactive properties. Secondly, we could not ascertain the mechanism of action which the essential oil stimulated in the cells. We only showed that *O. majorana* essential oil has a cytotoxic effect by causing membrane damaging and apoptotic effects; however, with further studies, this may be discovered. Thirdly, the effects of the main components of the essential oil on the cytotoxic effects of the essential oil were not ascertained within the scope of our study. The main components may be determining the cytotoxic effect of the essential oil. Building on the promising results we obtained from our study, further steps can be taken to investigate the effects and mechanisms of action of this essential oil *in vivo*. Thus, results that can lead to clinical studies can be obtained.

CONCLUSION

The results of our study show that *O. majorana* essential oil can be suggested as a natural herbal source in the production of new anticancer drugs, with the findings that it causes cytotoxic effects, membrane damage and apoptotic effects in A-549 and A-431 cells. Thus, new strategies that can be used in the treatment of lung and epidermoid carcinoma will be determined. The use of essential oils for the development of drugs for cancer treatment will also contribute to

the protection of human health. We believe that a better understanding of the intracellular mechanisms of essential oils obtained from other plants, similar to the essential oil obtained from *O. majorana*, will bring new strategies for the production of drugs used in cancer treatments.

MAIN POINTS

- *O. majorana* essential oil showed cytotoxic activity depending on concentrations and time on A-549 and A-431 cells according to both CellTiter-Blue® Cell Viability and MTT tests.
- *O. majorana* induces cytotoxicity on A-549 and A-431 cells in a membrane-damaging and apoptotic effects mediated manner.
- *O. majorana*'s anti-tumoral property suggests that it could be a potential source of lung and epidermoid carcinoma treatments.

ETHICS

Ethics Committee Approval: The study does not require ethics committee approval since it does not involve any human or animal subject.

Informed Consent: The study does not require informed since it does not involve any human or animal subject.

Peer-review: Externally peer-reviewed.

DISCLOSURES

Financial Disclosure: The author declared that this study had received no financial support.

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